Multivesicular bodies (MVB) are membranous cytoplasmic inclusions that are invariably associated with tombusvirus infections regardless of the virus species, the host, or the tissue examined. MVB are virus-induced structures since they are absent from tissues of healthy plants and are always present both in infected plants and protoplasts. MVB derive from peroxisomes in cells infected by a number of tombusviruses including cymbidium ringspot virus (CymRSV) and from mitochondria in cells infected by another tombusvirus, carnation Italian ringspot virus (CIRV). By using common restriction sites in full-length infectious clones, hybrid clones of these two viruses were constructed. In addition, a mutant of CIRV was prepared in which the protein encoded by the first open reading frame was shortened by 22 amino acids. All mutant transcripts were viable and infected Nicotiana benthamiana plants. Infected leaf tissue samples were collected, processed for thin sectioning, and observed in the electron microscope. The origin of MVB was shown to be under the control of the 5′ region of the viral genome. A sequence as short as about 600 nucleotides in ORF 1 contained the determinants for formation of MVB from peroxisomes or mitochondria.

The protein encoded by ORF 1 (33–36 kDa) does not contain sequence motifs equivalent to any known viral function, while the readthrough domain of ORF 2 contains the eight conserved motifs (PI–PVIII) which characterize the RNA-dependent RNA polymerase of supergroup II of the positive-strand RNA viruses (Koonin, 1991). Direct experimental evidence for the involvement of proteins encoded by ORFs 1 and 2 in replication has been provided (Dalmay et al., 1993a; Kollar & Burgyan, 1994; Scholthof et al., 1995a). The product of ORF 3 is the capsid protein. The proteins encoded by ORFs 4 and 5 are involved in the cell-to-cell and long-distance spread of virus in infected tissues (Dalmay et al., 1993a; Scholthof et al., 1993, 1995b).

One of the major cytopathological features of tombusvirus infections is the occurrence, in infected cells, of membranous cytoplasmic inclusions called multivesicular bodies (MVB) (Martelli et al., 1988). MVB consist of a main body surrounded by many spherical to ovoid vesicles measuring 80–150 nm in diameter. These vesicles contain fine fibrillar material consisting of double-stranded RNA (Russo et al., 1983; Di Franco et al., 1984). MVB incorporate [3H]uridine (Appiano et al., 1986) and appear in infected tissue before progeny virus is detected (Appiano et al., 1981). More recently, in cymbidium ringspot tombusvirus (CymRSV) infections, the proteins of the replicase complex were found to be associated with membranous cell...
Fig. 1. Diagrammatic representation of the genomes of CymRSV (shaded bars), CIRV (open bars) and hybrid clones. The common restriction sites used for the construction of hybrids are shown. For each clone, the type of organelle from which multivesicular bodies arise is indicated.

Components sedimenting at 30,000 g (Lupo et al., 1994). The same evidence was provided for tomato bushy stunt tombusvirus (TBSV) (Scholthof et al., 1995a). All this seems to provide evidence that MVB are the site of tombusvirus replication.

MVB originate by progressive vesiculation of the bounding membrane of cell organelles. Ultrastructural and cytochemical studies showed that, in cells of Nicotiana benthamiana and N. clevelandii systemically infected by CymRSV, TBSV, artichoke mottled crinkle (AMCV) or eggplant mottled crinkle (EMCV) tombusviruses, the MVB contained glycolate oxidase and were derived from peroxisomes (Russo et al., 1983; Martelli et al., 1984; M. Russo, unpublished results). Conversely, with carnation Italian ringspot tombusvirus (CIRV), visual and cytochemical evidence (e.g. detection of cytochrome oxidase in the developing MVB) suggested that MVB developed from mitochondria (Di Franco et al., 1984). The mitochondrial origin proved to be a highly consistent trait of CIRV MVB, which was independent of the host or type of host reaction (i.e. chlorosis, necrosis) (Russo et al., 1987).

In this paper, the extent to which the origin of MVB is governed by the viral genome was investigated by site-directed mutagenesis or by producing infectious chimeric
transcripts of CymRSV and CIRV, and examining *N. benthamiana* plants infected with mutant viruses.

**Methods**

- **Preparation of a full-length cDNA of CIRV.** CIRV propagation in *N. benthamiana*, virus purification and RNA extraction were as in Rubino *et al.* (1995). To obtain infectious *in vitro* transcripts, a full-length cDNA copy of CIRV genomic RNA was constructed using newly synthesized 5' and 3' terminal CIRV sequences and a clone containing approximately 3800 nt internal to the complete sequence of the CIRV genome (Rubino *et al.*, 1995).

  The 5' region was cloned by priming first-strand cDNA synthesis with the oligonucleotide 5' CCTGGGAGGTTACGTA 3', complementary to nt 630-647 of the CIRV genomic RNA sequence. Hybrid RNA-DNA molecules were melted at 100 °C for 1 min, and the cDNA was amplified by 35 cycles of PCR. The second-strand primer was 5' ATCGATAATACGACTCACTATAGGAA.ATATCTCAGGATT Y, complementary to nt 630-647 of the CIRV genomic RNA sequence, and five bases contributing to the formation of a ClaI restriction site. The melting, annealing and polymerizing steps were carried out at 94 °C (1 min), 45 °C (1 min) and 72 °C (1 min), respectively. The last polymerization step was extended by 10 min. The major PCR product of the expected size (669 bp) was eluted in 1% low melting point agarose, made blunt-ended with Klenow enzyme, ligated to Smal-digested, dephosphorylated pUC18 and cloned in *Escherichia coli* strain DH5α.

  The 3' region was cloned by priming first-strand cDNA synthesis with the oligonucleotide 5' GGCGCTATTTCTGCATG 3', complementary to the last 19 nucleotides of the genomic RNA sequence. The cDNA was amplified by PCR using this oligonucleotide and the oligonucleotide 5' GGCGCTATTTCTGCATG 3', homologous to nt 3815-3833 in the CIRV genomic RNA sequence. The PCR product (946 bp) was cloned into Smal-digested and dephosphorylated pUC18 so that a Smal site was generated precisely at the 3' terminus of the CIRV sequence.

  For full-length clone construction, the 5' and 3' regions were fused to the 3800 bp CIRV clone by using the SplI and EcoRI restriction sites, respectively, which are present once within the inserts (nt positions 571 and 4016 in the CIRV genomic sequence, respectively) and vector sequences.

- **Construction of mutant clones.** The exchange of sequences between CIRV and CymRSV genomes was carried out using full-length clones of both CIRV, described above, and CymRSV (Burgyan *et al.*, 1990; Dalmay *et al.*, 1993b). Hybrid molecules were constructed using restriction sites common to both clones (*Fig.* 1). Briefly, the two clones were digested with a pair of restriction enzymes both cutting in the viral sequence, or one in the viral sequence and the other in the pUC18 polylinker. Donor and acceptor molecule (i.e. the one carrying vector sequence) fragments were eluted after electrophoresis in low molecular weight agarose, mixed and ligated with T4 DNA ligase (BioLabs). Recombinant DNA was cloned in *E. coli* strain DH5α and identified by restriction analysis and DNA sequencing with T7 DNA polymerase (Sequenase, US Biochemical).

  Other mutants were prepared by modifying the sizes of ORF 1 and/or the 5′ leader sequence. Mutant C80 was constructed by site-directed mutagenesis (Kunkel *et al.*, 1987) of the CIRV full-length clone using the oligonucleotide 5' TCAAACCTCGATAGAACAA 3′, complementary to nt 70-90 of CIRV (the substituted nucleotide is underlined). This mutation modified the AUG to AUC at position 80 inactivating the initiation codon of ORF 1. Mutagenesis was performed using a Mutagen Kit (Bio-Rad) following the manufacturer’s instructions. Mutant DNA was sequenced using the Sequenase protocol.

  Mutant 125/3 was produced by replacing the first 98 nt of CIRV RNA with the first 114 nt of CymRSV using the AccI sites at these positions in CIRV and CymRSV, respectively. Reciprocal mutant C80/G11 was constructed by replacing the first 114 nt of CymRSV with the first 98 nt of CIRV mutant C80.

- **In vitro transcription and inoculation to plants.** Infectious RNAs were obtained from 2 μg plasmid DNAs, linearized with Smal, using T7 RNA polymerase and a T7 transcription kit according to the manufacturer’s (BioLabs) instructions. For inoculation, RNA was diluted with an equal volume of inoculation buffer containing 1% Celite and 1% bentonite (Heaton *et al.*, 1989) to a final concentration of about 75 μg/ml and was applied to three leaves (10 μl/leaf) of *N. benthamiana* plants with a sterile glass spatula.

- **Protein extraction and Western blot.** Proteins were extracted from infected tissues, fractionated by differential centrifugation and SDS–PAGE on a 12% gel (Laemmli, 1970), transferred to Immobilon-P nylon membranes (Millipore) and exposed to an antiserum to the 33 kDa protein encoded by ORF 1 of CymRSV, as previously described (Lupo *et al.*, 1994). Antigen–antibody complexes were visualized by incubation with alkaline phosphatase-linked goat anti-rabbit IgG, followed by incubation with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and p-nitro blue tetrazolium chloride (NBT) (Sigma).

- **Electron microscopy.** Tissues were sampled from the upper leaves of systemically infected plants 1 week after inoculation, and processed as described by Martelli & Russo (1984), i.e. fixation in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.4), post-fixation in 1% osmium tetroxide for 2 h at 4 °C, staining overnight in 0.5% aqueous uranyl acetate at 4 °C, dehydration in a graded ethanol series, and embedding in Spurr’s medium. Thin sections were stained with lead citrate and viewed with a Philips 201 C electron microscope.

**Results and Discussion**

**Construction and analysis of hybrid clones**

As shown in *Fig.* 1, most of the hybrid clones could be constructed by exchanging complete ORFs between CymRSV and CIRV genomes. This was not so for clones 81/8, 79/8, 107/1, 93/4, 113/7 and 113/2 in which the only convenient restriction site (*NsiI*) was 144 nt upstream of the termination codon of ORF 2; however, the C-terminal region of CIRV and CymRSV polymerases differs only in two amino acids at positions 489 (leucine to valine) and 505 (glutamic acid to arginine), respectively. Clones 109/1 and 101/2 contained the 5′ non-coding region and the N-terminal two-thirds of ORF 1 of CymRSV or CIRV in the context of CIRV or CymRSV, respectively; the sizes of predicted chimeric products of ORF 1 of clones 109/1 (32771 Da) and 101/2 (36658 Da) were almost identical to the corresponding products of CymRSV (32984 Da) and CIRV (36445 Da), respectively.

*In vitro*-synthesized RNAs from the full-length CIRV cDNA clone and all hybrid clones were infectious when inoculated to *N. benthamiana* plants. Northern blot analysis of infected tissues showed the presence of genomic and subgenomic
Fig. 2. Expression of ORF 1-encoded protein in systemically infected leaves of *N. benthamiana* inoculated with CIRV, CymRSV or a selection of hybrids as indicated. Proteins were sedimented at 30000 g and equivalent amounts of each sample were loaded for immunoblot analysis. Arrows correspond to the sizes in kDa of the protein products of CIRV (36 kDa) and CymRSV (33 kDa).

RNAs of the expected sizes (not shown). Western blot analysis showed that the mutants replicated well and that the proteins encoded by ORF 1 of the mutant clones were of the expected sizes (Fig. 2).

**Cytopathology of infected tissues**

The ultrastructural modifications of cells infected by CymRSV and CIRV have been described (Di Franco et al., 1984; Russo et al., 1983). Analysis of tissues infected with *in vitro* synthesized RNA from full-length clones of these two viruses confirmed that MVB develop from peroxisomes in CymRSV-infected (Fig. 3) and mitochondria in CIRV-infected (Fig. 4) tissues.

The organellar origin of the MVB, in tissues systemically infected with chimeric *in vitro* transcripts, was determined by

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**Fig. 3.** Origin of MVB in *N. benthamiana* infected with *in vitro*-synthesized CymRSV RNA. A CymRSV-infected mesophyll cell showing MVB (arrowed) derived from peroxisomes is shown (scale bar, 0.5 μm). The inset shows a vesiculating peroxisome at the initial stage of transformation into an MVB (scale bar, 0.2 μm).
the source of the 5' region, regardless of whether the rest of the molecule was completely derived from the heterologous virus (Fig. 1a, b), or was made up of sequences from both viruses (Fig. 1c). Only an occasional cell infected with some hybrids with a CIRV 5'-end (116/6, 79/8, 93/4) had a few peroxisomes showing light peripheral vesiculation (i.e. involving only part of the bounding membrane), in addition to mitochondria-derived MVB. These results were variable from experiment to experiment and were not further investigated.

Infection with hybrids 88/5, 97/5, 107/1 and 93/4 indicated that determinants for the origin of MVB are located in the region comprising the 5' non-coding leader sequence and the pre-readthrough portion (ORF 1) of the viral polymerase (Fig. 5a-d), while analysis of tissues infected with clones 109/1 and 101/2 allowed for mapping the organellar origin of MVB up to the 5' non-coding sequence and about two-thirds of ORF 1 (Fig. 5e, f).

To find out whether the 5' leader or the 5'-most ORF 1-coding sequence, or both, were responsible for the specific cytopathological behaviour, three more clones were prepared (Fig. 6). Clone C80 was obtained by site-directed mutagenesis, converting the AUG start codon of wild-type CIRV (position 78–80) to AUC, so that the first available AUG was at position 144–146. The protein encoded by ORF 1 of this clone had a predicted size of 34 kDa instead of 36 kDa for wild-type CIRV (Rubino et al., 1995).

In clone 125/3, 114 nt of the leader sequence of CymRSV (160 nt; Grieco et al., 1989) were substituted for 98 nt of the CIRV sequence (i.e. 77 nt of the leader and 21 nt of the ORF 1 sequence; Rubino et al., 1995). Since the first AUG codon in this clone was located at position 160, the leader sequence became 159 nt long, being composed of 114 nt from CymRSV and 45 nt from CIRV. The latter is 65% identical to the equivalent sequence in CymRSV RNA (Rubino et al., 1995). The proteins encoded by ORF 1 of both clones C80 and 125/3 had the same size, but the 5'-leader sequence of clone C80 was completely derived from CIRV except for the conversion of G at position 80 to C.

Fig. 4. Origin of MVB in N. benthamiana infected with in vitro-synthesized CIRV RNA. A CIRV-infected mesophyll cell showing MVB (arrowed) derived from mitochondria is shown (scale bar, 0.5 μm). The inset shows a vesiculating mitochondrion at the initial stage of transformation into an MVB (scale bar, 0.2 μm).
Fig. 5. MVB derived from modified peroxisomes in N. benthamiana cells infected with hybrid clones 88/5 (a), 107/1 (c) and 109/1 (e), and from modified mitochondria in cells infected with hybrid clones 97/5 (b), 93/4 (d) and 101/2 (f) (scale bar, 0.2 μm).

Fig. 6. Mutant clones of CIRV (C80 and 125/3) and CymRSV (C80/G11) genomes compared with wild-types. Only the 5′-leader sequence (continuous or broken lines) and part of ORF 1 are shown. M indicates methionine codons. In clones C80 and 125/3 the first in-frame AUG codon of CIRV ORF 1 was eliminated by mutagenesis.

The third clone (C80/G11) had a 5′-leader composed in part of the leaders of CIRV and CymRSV, the rest being derived from CymRSV sequence.

In vitro-synthesized RNAs from these three clones were as infectious as the wild-type viruses and the other hybrid clones. Western blot analysis showed that the ORF 1 product had the...
expected size (34 kDa), with no detectable reversion to wild-type CIRV (not shown).

Thin sections from leaf tissues infected with transcripts from clones C80 and 125/3 showed that the MVB were derived from mitochondria. Thus, the N-terminal 22 amino acids of CIRV ORF 1 are not responsible for determining the origin of MVB (and, incidentally, are dispensable for replication). Conversely, cells infected with transcripts from clone C80/G11 had MVB derived from modified peroxisomes as occurs in CymRSV infections. In conclusion, the results obtained with clones C80, 125/3 and C80/G11 suggest that the 5' leader sequence had no influence in determining the cytopathological behaviour of CIRV and CymRSV.

This study has shown that the major cytopathological alterations of tombusvirus-infected cells are under the control of the virus genome. Whether MVB consist of transformed peroxisomes or mitochondria depends on the composition of the N-proximal region of ORF 1 of the infecting virus (amino acids 1-189 and 23-223 in CymRSV and CIRV, respectively).

Although direct proof of the association of viral RNA synthesis with MVB is lacking, it is conceivable that virus replication takes place within the MVB. Hydrophathy profiles of the proteins encoded by ORF 1 of CIRV and CymRSV show a hydrophobic domain flanked by hydrophilic regions (Fig. 7). This domain (between amino acids 80-150 and 97-184 in CymRSV and CIRV, respectively) resides in the N-proximal sequence, which is the least conserved between CymRSV and CIRV (about 60% amino acid sequence identity), whereas the C-terminal amino acids show 90% sequence identity (Rubino et al., 1995). Exchanging the N-proximal 189/223 amino acids between CymRSV and CIRV (clones 109/1 and 101/2) changed the origin of the MVB, whereas deletion of the N-terminal 22 amino acids of the CIRV ORF 1 sequence had no effect (clones C80 and 125/3).

We conclude, therefore, that the hydrophobic domain of the pre-readthrough portion (ORF 1) of the tombusvirus replicase (ORF 2) (Fig. 7) is responsible for the selective association of the viral replicase with membranous vesicles, determining which cell organelle contributes the membranous backbone for virus replication.

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